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P-Glycoprotein Expression in Cartilaginous Tumors

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Background and Objectives: Malignant cartilage tumors demonstrate chemotherapeutic resistance through undetermined mechanisms. P-glycoprotein is the protein product of the multiple drug resistance gene 1 (MDR-1) and confers multidrug chemotherapeutic resistance in a variety of malignancies.

Methods: MDR-1 expression was examined in 55 benign and malignant cartilage tumor specimens by immunohistochemistry using C219, C494, and JSB-1 antibodies, and by in situ hybridization with an MDR-1 specific oligonucleotide cDNA probe.

Results: Constitutive expression of P-glycoprotein was observed in all benign and malignant cartilage tumor specimens with a similar pattern of immunohistochemical staining present with all three antibodies. In benign tumors and low grade chondrosarcomas, the staining pattern was weak to intermediate and localized to clusters of cells. However, higher grade tumors (Grade II and III) expressed P-glycoprotein in a higher percentage of cells and with more intense staining. P-glycoprotein expression was absent in normal human articular cartilage, but was focally present in costal and growth plate cartilage. The immunohistochemistry results were confirmed by in situ hybridization in 10 cases.

Conclusions: P-glycoprotein is expressed constitutively in cartilaginous tumors, with greatest expression in high grade malignancies. The findings may account for the resistance of cartilage tumors to chemotherapeutic agents. *J. Surg. Oncol. 1997;65:95–105.*

KEY WORDS: **MDR-1; cartilage; chondrosarcoma; P-glycoprotein; sarcoma**

INTRODUCTION

Chondrosarcoma is a neoplastic proliferation of cartilaginous tissue that can be osseous or extraosseous in origin and can arise de novo or secondary to malignant transformation of a benign lesion [1–4]. Malignant cartilaginous neoplasms have distinct clinical, therapeutic, and prognostic features [2,4–8]. Low grade tumors have histological features that overlap with their benign counterparts [9–11]. Metastases rarely occur in low grade tumors and typically appear late, often after local recurrence. High grade tumors demonstrate more aggressive clinical and biological behavior with frequent metastases, most commonly to the lung [2,4–7]. Both low and high grade chondrosarcomas are resistant to chemotherapy and radiation, making surgery the mainstay of treatment [4,12–15]. Current treatment consists of wide excision

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[4,12–14], but an understanding of the biologic mechanisms underlying the chemoresistance of these tumors may facilitate the development of treatment strategies for patients with disseminated disease or at high risk for metastasis [2,5–7]. The purpose of this study was to investigate multiple drug resistance gene expression in benign and malignant cartilage tumors as a possible explanation for the intrinsic resistance of chondrosarcomas to chemotherapy.

One mechanism of chemotherapeutic resistance in many malignancies is P-glycoprotein, the protein product of the multiple drug resistance gene (MDR-1) [16–18]. This ATP-dependent membrane protein pump removes a wide spectrum of cytotoxic drugs from tumor cells [16–20]. P-glycoprotein is present in a number of normal tissues, such as brain, kidney, and muscle, as well as in a variety of human malignancies, including primary osseous and soft tissue sarcomas [16–27]. The functional importance of P-glycoprotein is evident in both hematopoietic malignancies and pediatric soft tissue sarcomas, which are otherwise extremely sensitive to chemotherapeutic agents [21,28]. Chan et al. [21] found P-glycoprotein expression in all nine patients who had relapse of a pediatric soft tissue sarcoma, whereas only one relapse occurred in a group of 20 patients with P-glycoprotein negative tumors. Recent studies have demonstrated chemotherapeutic resistance, an increased incidence of metastasis, and a poorer prognosis in osteosarcomas expressing P-glycoprotein [25–27].

The presence of P-glycoprotein was determined by immunohistochemistry with three monoclonal antibodies: (1) C219, which detects a carboxy-terminal intracellular epitope and reacts with the products of the human MDR-1 and MDR-2 genes [29], (2) C494, which recognizes and reacts specifically with P-glycoprotein, the product of the MDR-1 gene [30,31], and (3) JSB-1, which reacts with a separate, conserved cytoplasmic epitope present on P-glycoprotein [32]. In situ hybridization studies utilizing a digoxigenin labeled oligonucleotide probe complementary to the P-glycoprotein m-RNA sequence were performed to confirm the expression of the MDR-1 gene in these tissues.

MATERIALS AND METHODS

Histologic Specimens

Cases of chondrosarcoma, osteochondroma, enchondroma, and chondroblastoma were retrieved from the surgical pathology files of Strong Memorial Hospital. Fifty biopsy and resection specimens from 39 patients collected between 1979 and 1992 were studied. In addition, four low grade chondrosarcomas and one enchondroma were obtained prospectively for comparison of the results of immunohistochemical staining on frozen and paraffin-embedded sections. Samples of normal articular, costal, and growth plate cartilage were obtained from surgical specimens. Tissues were fixed and decalcified in a mixture of formaldehyde and formic acid (Surgipath Decalcifier, Richmond, IL), prior to paraffin embedding and sectioning, which in our experience is an effective method for preserving protein antigenicity and nucleic acids in decalcified bone specimens [33].

Immunohistochemistry

Immunohistochemistry for P-glycoprotein was performed by the biotin/streptavidin method [34,35] using the monoclonal antibodies C219, C494, and JSB-1 (Signet Laboratories, Dedham, MA) directed against different epitopes of the MDR-1 gene product [31,36]. Sections of normal kidney, liver, and adrenal gland were used as positive control tissues, since they express P-glycoprotein constitutively in specific cells [17]. Negative controls were performed by omission of the primary antibody. Immunostaining for the vimentin intermediate filament was used to demonstrate preservation of immunoreactivity in the experimental tissues.

Endogenous peroxidase activity was consumed with hydrogen peroxide and tissue sections were rinsed in phosphate-buffered saline (PBS). Specimens were incubated with the primary antibody (1:10 monoclonal anti-MDR-1 C219, 1:300 C494, or 1:20 JSB-1) overnight at 4°C; 0.5% PBS-bovine serum albumin was used as diluent for the antibodies and was applied alone as the negative control for each antibody. The secondary antibodies (Vector Laboratories, Burlingame, CA, used at 1:200 dilution) were incubated with the sections for 30 minutes. Horseradish peroxidase-streptavidin conjugate (Jackson Immunological Research Laboratories, W. Grove, PA, used at 1:1000 dilution) was applied and allowed to incubate for 30 minutes. The chromagen, 3-amino-9-ethylcarbazole (Zymed Laboratories, San Francisco, CA) was applied to each section. Sections were then counterstained with Mayer's hematoxylin and mounted with permanent aqueous media. Immunohistochemical staining was scored on a scale of 0–4+ according to the scoring system outlined in Table I. Categories for stronger staining in smaller percentages of cells were not included since this phenomenon was not observed in any specimens. All sections were analyzed using the C219 antibody, whereas 9 of the 23 benign lesions, 8 of the 11 low grade chondrosarcomas, and 8 of the 12 high grade chondrosarcoma specimens were also evaluated with C494. Paired frozen and formalin-fixed, paraffin-

TABLE I. Immunostaining/In Situ Hybridization Grading Scale

Grade 0	No immunoreactivity
Grade 1+	Weak staining of up to 10% of cells
Grade 2+	Intermediate staining of up to 30% of cells
Grade 3+	Intermediate staining of up to 60% of cells
Grade 4+	Strong staining of >60% of cells

embedded sections from four prospectively obtained low grade chondrosarcomas and one enchondroma were studied with C219, C494, and JSB-1 to confirm both the consistency and the specificity of the immunoreactivity reported.

In Situ Hybridization

In situ hybridization was carried out using a modified digoxigenin-11-dUTP labeled nonradioactive technique (Genius™ kit, Boehringer-Mannheim, Indianapolis, IN) with immunodetection using an antidigoxigenin antibody. The modified procedure, which has been optimized for analysis of skeletal tissues, has been used extensively in our laboratory and previously published [33]. A 30-base oligonucleotide probe complementary to MDR-1 mRNA (antisense probe) and a 30-base control sense probe were constructed, and the sequences evaluated by a Wisconsin Genbank search to confirm lack of homology with other genes. A 30-base poly-dT probe was used as a positive control to demonstrate preservation of mRNA in tissue sections [33]. Probes were end-labeled with digoxigenin-11-dUTP, using the Boehringer Mannheim Biochemicals DNA tailing kit as previously described [33]. The tissue sections were deproteinized with HCl, sequentially digested with proteinase K and hyaluronidase, and then fixed in 4% paraformaldehyde. Sections were rinsed in PBS and acetylated to reduce background staining. The specimens were then incubated with prehybridization solution for 1 hour, rinsed in saline-sodium citrate (SSC), and the digoxigenin-11-dUTP labeled MDR-1 antisense and sense oligonucleotides were applied and incubated overnight at 37°C. The slides underwent posthybridization stringency washes in decreasing concentrations of SSC, were rinsed in buffer 1 (tris-HCl, NaCl, pH 7.5), and were preblocked in normal sheep serum. Alkaline phosphatase conjugated antibody was added followed by a wash in buffer 1. Freshly prepared color solution (Nitro Blue tetrazolium) was added to each section and maintained in darkness for 10–12 hours. The color reaction was stopped and the specimens were dehydrated, rinsed in xylene, and prepared for microscopic examination. Staining on in situ hybridization sections was scored on a scale of 0–4+, similar to the immunostaining scoring system outlined in Table I.

Statistics

Statistical comparisons of the immunostaining scores were performed between low grade (grade I), intermediate grade (grade II), and high grade (grade III) malignancies by analysis of variance. Since only the C219 antibody was utilized in all sections, statistical comparisons are presented for the staining score of this antibody only. However, a statistically significant difference at the $P < 0.001$ level is also present for the C494 antibody.

RESULTS

Clinicopathologic Findings

A total of 50 cartilage tumor specimens from 39 patients, including 23 men and 16 women, were retrospectively reviewed. The diagnoses included 16 enchondromas, 5 osteochondromas, 2 chondroblastomas, and 27 primary or recurrent chondrosarcomas. The mean age at diagnosis was younger in patients with benign tumors (28.6 ± 3.2 years) or grade I chondrosarcomas (29.1 ± 2.6 years), as compared to high grade chondrosarcomas (51.6 ± 6.8 years). Including primary and recurrent tumors, there were 11 grade I, 10 grade II, and 6 grade III chondrosarcomas.

The specimens were from the axial skeleton in 9 cases, the facial bones in 4 cases, the lung in 4 cases, and the appendicular skeleton in 33 cases. Follow-up of these patients was a minimum of 2 years. In the 21 benign tumors, no metastasis or local recurrence developed. In contrast, 1 of 6 grade I chondrosarcomas had multiple local recurrences, whereas 2 of the 12 grade II or grade III chondrosarcomas had a local recurrence and distant metastasis developed in 5 other patients. The two patients with mesenchymal chondrosarcoma (cases 38 and 39) received chemotherapy and radiation for their tumors.

Immunohistochemical Findings

Validation of immunohistochemistry in paraffin-embedded cartilage specimens. In order to confirm that the C219, C494, and JSB-1 antibodies are immunoreactive in surgical specimens from our pathology files, immunohistochemistry was performed on paired frozen and formalin-fixed, paraffin-embedded sections from four low grade chondrosarcomas and one enchondroma. In each of the tumors, chondrocyte immunoreactivity was identified, and a similar pattern of staining was observed with each of the antibodies in both frozen and paraffin embedded tissue (Table II). The staining pattern and intensity were similar with each of the antibodies, with clusters of positive cells intermixed with P-glycoprotein negative cells (Fig. 1A–C).

Control Tissues

Immunohistochemistry was performed on cartilage tumors and control tissues using C219, C494, and JSB-1 monoclonal antibodies. Positive controls included sections of kidney, adrenal gland, and liver, which exhibited characteristic staining patterns with all three antibodies. Kidney sections demonstrated immunoreactivity in the proximal convoluted tubules, whereas adrenal sections showed cortical staining and liver sections showed a canalicular pattern of staining and bile duct staining. The pattern of staining was similar with the three separate antibodies. Immunostaining with C219 in kidney is shown in Figure 1D. In contrast, no staining was observed in any of the control specimens when the primary

TABLE II. Correlation of the Immunoreactivity for C219, C494, and JSB-1 in Formalin-Fixed vs. Frozen Tissue*

Prospective case #	Diagnosis	Formalin-fixed tissue			Fresh frozen tissue			In situ hybridization		
		C219	C494	JSB-1	C219	C494	JSB-1	poly-dT	MDR	sense MDR
1	CS Grade I	3+	3+	2+	2+	2+	2+	4+	3+	—
2	CS Grade I	1+	1+	1+	1+	1+	1+	—		
3	CS Grade I	1+	1+	2+	1+	1+	1+	—		
5	CS Grade I	1+	1+	1+	1+	1+	1+	—		
5	EC (cellular)	3+	3+	3+	3+	2+	3+	—		

*C219, C494, and JSB-1 immunoreactivity was evaluated without prior knowledge of the histologic diagnosis. The lesions were graded semiquantitatively on a scale of 0–4+ as described in Table I. Case #1 had adequate preservation of mRNA and therefore in situ hybridization was done for MDR-1. The other cases were negative for the poly-dT, suggesting that there was degradation of cellular mRNA and therefore further in situ hybridization was not done. CS = chondrosarcoma; EC = enchondroma; MDR-1 = multiple drug resistance gene 1.

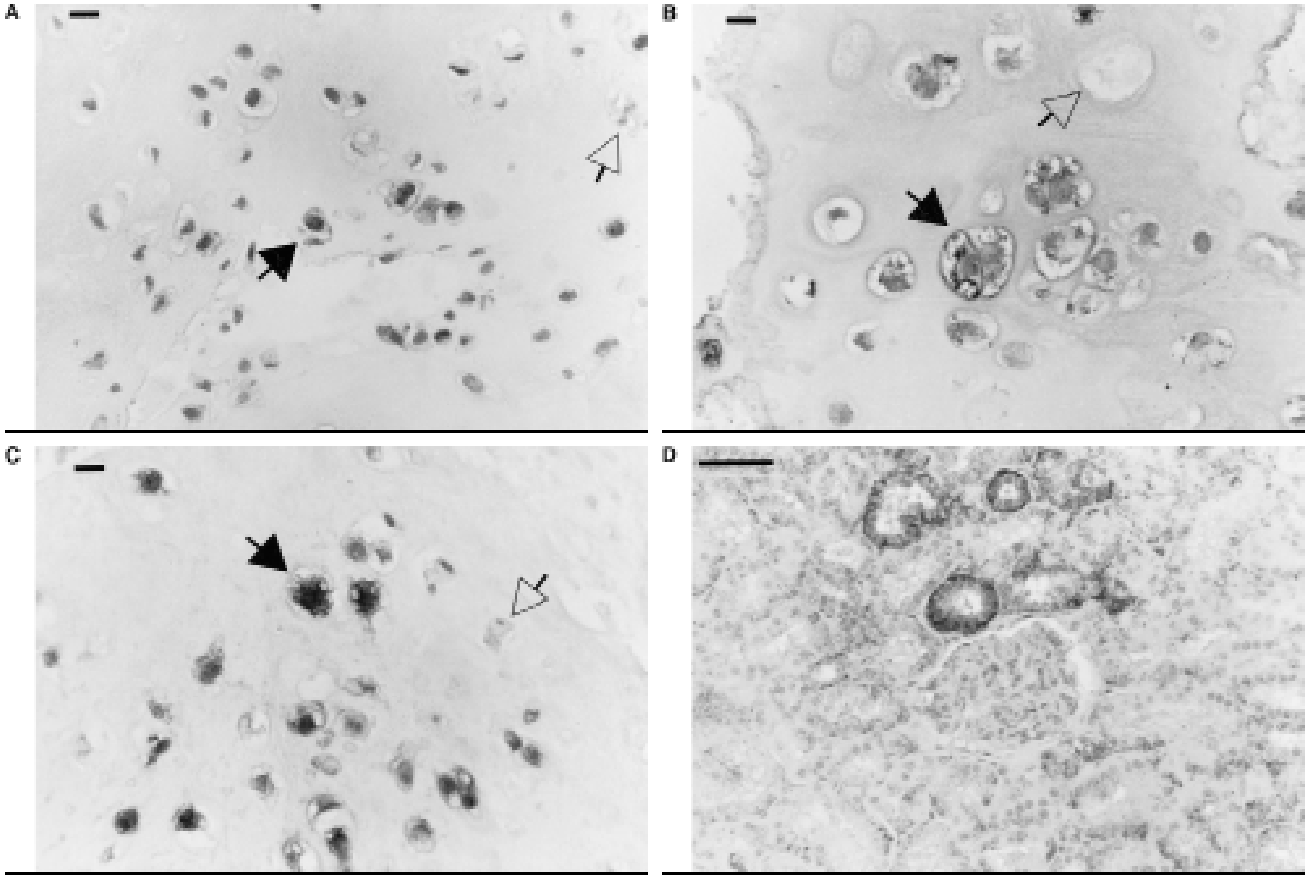

Fig. 1. Immunostaining of a low grade chondrosarcoma for P-glycoprotein expression. Sections of a low grade chondrosarcoma (Table II, Case 1) were immunostained as described in Materials and Methods using the C219 antibody (**A,** original magnification = 400×, bar = 10 μm); the C494 antibody (**B,** original magnification = 400×, bar = 10 μm); and the JSB-1 antibody (**C,** original magnification = 400×, bar = 10 μm). P-glycoprotein negative cells are indicated by closed arrows and P-glycoprotein positive cells by solid arrows. Normal human kidney immunostained with C219 antibody, showing expression in the proximal convoluted tubules (**D,** original magnification = 200×, bar = 50 μm).

antibody was omitted. Additionally, nearly identical immunoreactivity was observed on frozen sections and fixed tissues when stained for each of the three antibodies.

Normal Cartilage

Normal human cartilage, including articular, growth plate, and costal cartilage, was examined for P-glycoprotein expression with both C219 and C494 antibodies. P-glycoprotein expression was absent in articular cartilage (data not shown). Focal, weak staining for P-glycoprotein was present in the hypertrophic cells of growth plate, near the mineralizing front, and to a greater degree in the chondrocytes of hyaline rib cartilage (Fig. 2).

Benign Tumor Cartilage

Immunoreactivity to P-glycoprotein was present in each of the benign tumors (Table III). In general, the

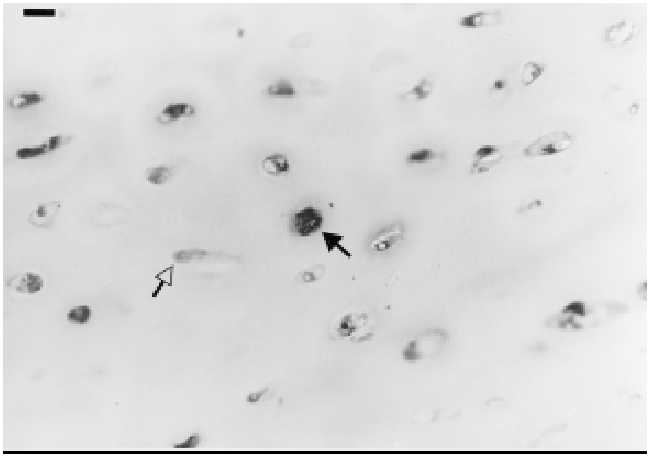

Fig. 2. Immunostaining of normal human rib hyaline cartilage for P-glycoprotein. Sections of rib hyaline cartilage were immunostained as described in Materials and Methods using the C219 antibody. P-glycoprotein negative cells are indicated by closed arrows and P-glycoprotein positive cells by solid arrows. Original magnification = 400×, bar = 10 μm.

staining was weak to intermediate with a minority of the cells exhibiting immunoreactivity to P-glycoprotein (immunostaining score 1.75 ± 0.16). In osteochondromas, immunoreactivity localized to cells that had a more hypertrophic appearance and were located near the ossifying regions of the cartilaginous cap (Fig. 3A), resembling the pattern of expression observed in growth plate. In enchondromas, staining was present within scattered clusters of cells intermixed with P-glycoprotein negative cells (Fig. 3B). In chondroblastomas, staining was present in chondroblasts primarily in areas of cartilaginous matrix formation (Fig. 3C). Similar patterns of immunoreactivity were present with both antibodies, although staining was more intense with C494, particularly in chondroblastomas. This suggests that C494 epitopes may be more accessible for antibody binding in chondroblastomas under the fixation and immunohistochemical conditions used in the present study.

Malignant Cartilage

Whereas all malignant cartilage tumors exhibited P-glycoprotein expression, the pattern of staining varied in the low and high grade lesions (Table IV). The low grade chondrosarcomas demonstrated weak to intermediate staining in clusters of cells in a pattern similar to that observed in benign enchondromas (Fig. 4A,C). In contrast, more intense staining and a higher percentage of P-glycoprotein positive cells were observed in the high grade chondrosarcomas (Fig. 4B,D). An intermediate or strong pattern of staining with either C219 or C494 was present in 14 of 16 high grade tumor specimens. As with the benign lesions, similar immunostaining patterns were observed with the two antibodies, although the intensity of the staining occasionally varied.

The immunostaining score was significantly different between low grade (1.33 ± 0.19), intermediate grade (1.90 ± 0.34), and high grade (3.00 ± .52) chondrosarcomas ($P < 0.01$), suggesting a higher level of expression in the more malignant tumors. In four patients, tissue from both primary and metastatic or recurrent tumors was available for review. In the two patients who had not received chemotherapy, P-glycoprotein staining in the metastatic or recurrent tumor was the same (≠28) or less (≠30), as compared to that in the primary lesion. In contrast, in the two cases of mesenchymal chondrosarcoma in which chemotherapy had been administered (≠38 and ≠39), higher levels of P-glycoprotein staining occurred in the metastatic lesions, suggesting the possible induction of P-glycoprotein in these tumors, or alternatively, greater expression in association with metastatic tumor progression (Table IV).

TABLE III. Immunohistochemical Data: Benign Cartilage Lesions*

Patient	C219	C494
Osteochondromas		
1	1	2+
2	2	2+
3	3	1+
4	4	2+
5	5	1+
Chondroblastomas		
6	1+	3+
7	1+	4+
Enchondromas		
8	2+	2+
9	2+	1+
10	1+	
11	2+[a]	1+[a]
12	2+	1+
13	1+	1+
14	2+	
15a	2+	1+
15b	2+	
16	4+[a]	
17a	1+	
17b	1+	1+
18	2+	
19	1+	
20	1+	
21	3+[a]	

*Each patient has been given a separate number. If more than one specimen was studied for a given patient (i.e., biopsy of lesion, then subsequent excision), the consecutive specimens were labeled, a, b, c, etc. C219 and C494 immunoreactivities were evaluated without prior knowledge of the histologic diagnosis. The lesions were graded semiquantitatively on a scale of 0–4+ as described in Table I.
[a]Indicates cases in which staining was localized to the cellular areas of the lesion.

In Situ Hybridization Findings

To confirm the immunohistochemical findings, a 30-base oligonucleotide probe complementary to MDR-1 was used for in situ hybridization on 10 specimens (nine malignant, one benign). On Northern blots, this probe

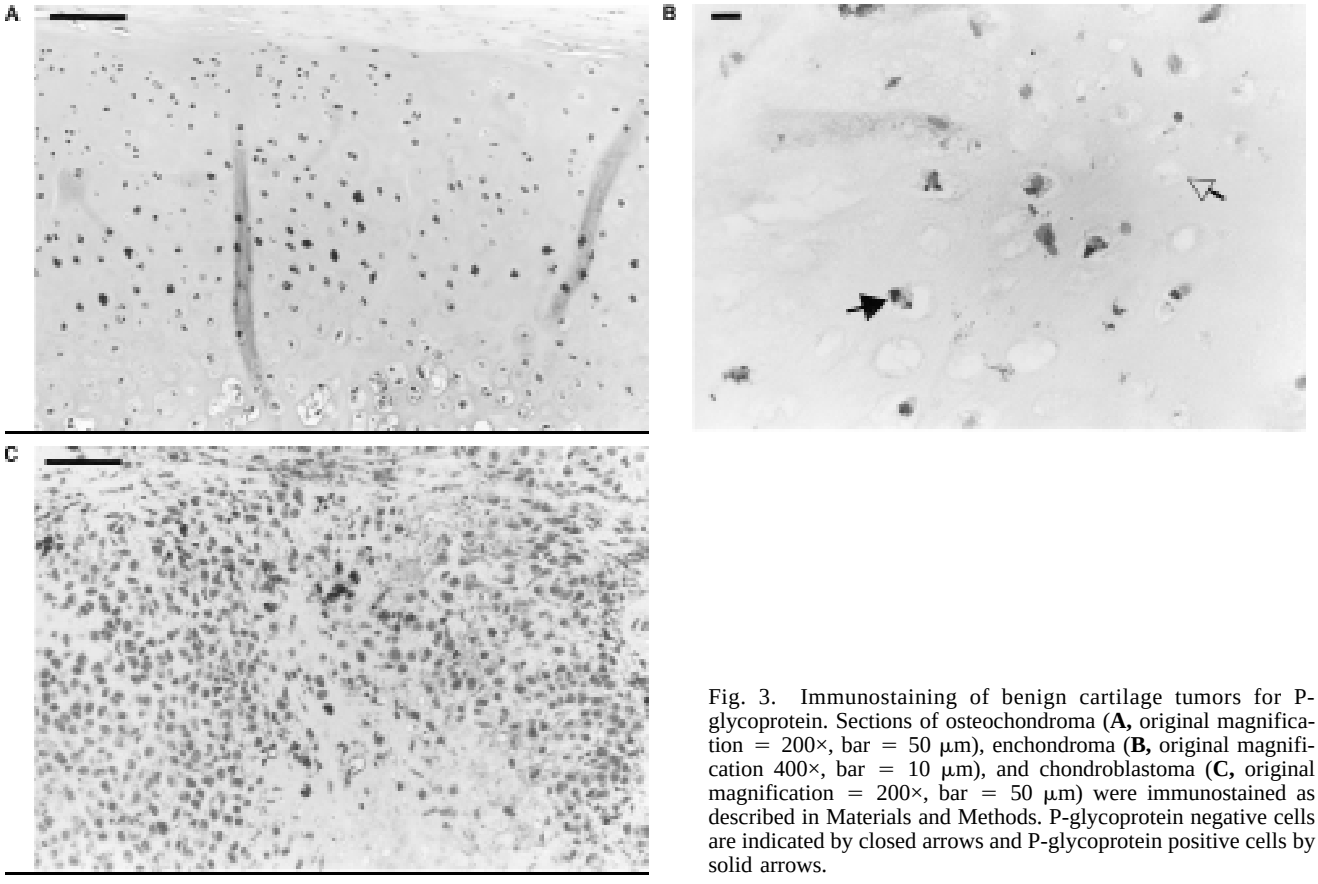

Fig. 3. Immunostaining of benign cartilage tumors for P-glycoprotein. Sections of osteochondroma (**A,** original magnification = 200×, bar = 50 μm), enchondroma (**B,** original magnification 400×, bar = 10 μm), and chondroblastoma (**C,** original magnification = 200×, bar = 50 μm) were immunostained as described in Materials and Methods. P-glycoprotein negative cells are indicated by closed arrows and P-glycoprotein positive cells by solid arrows.

hybridizes to a 4.5 kB transcript consistent with MDR-1, and correlates with immunohistochemical localization of P-glycoprotein in osteosarcoma [26]. Preservation of mRNA was confirmed by hybridization with a 30-base digoxigenin labeled poly dT probe (data not shown), whereas a nonsense probe served as a negative control. Messenger RNA expression for P-glycoprotein was present in all 10 tumors (Table V). Consistent with the immunohistochemistry findings, the mRNA expression was greater in the higher grade chondrosarcomas compared to low grade malignant tumors. In both cases, minimal background staining is present with the nonsense probe (Fig. 5A,C), whereas the tissue sections demonstrate strong staining with the antisense probe complementary to the MDR-1 mRNA sequence (Fig. 5B,D). Likewise, the pattern of mRNA localization also demonstrated clusters of positive cells intermixed with P-glycoprotein negative cells.

DISCUSSION

Chondrosarcoma is a rare mesenchymal neoplasm in which the clinical behavior ranges from locally aggressive tumors with minimal metastatic potential to high grade malignancies with a strong propensity for metastasis [2,5–9]. Surgery remains the mainstay of treatment, with a goal of eradication of local disease [4]. Prognosis is worse for patients who have experienced local recurrence [7] and dismal for those with metastases [5–7]. Treatment with chemotherapy or radiation does not significantly improve outcome or prolong survival in patients with disseminated disease [5,37,38]. The present study demonstrates the expression of P-glycoprotein in all 55 benign and malignant cartilage tumors examined, indicating that constitutive expression of this membrane pump may partly account for the well-recognized insensitivity of chondrosarcoma to chemotherapy.

P-glycoprotein is an adenosine triphosphate (ATP)-dependent drug efflux pump homologous with several mammalian and bacterial transport proteins [16,17,39]. Localization of P-glycoprotein to tissues with secretory function, such as the gastrointestinal tract, kidney, adrenal, endometrium, and liver, suggests that this protein may have a normal role in molecular transport [16,17,40,41]. In this study, P-glycoprotein expression was absent in articular cartilage but was identified in normal cartilage, which characteristically undergoes endochondral ossification. Similar to our findings, Mangham et al. [42] have recently immunolocalized P-glycoprotein expression in the growth plate to hypertrophic chondrocytes and have confirmed these findings by PCR amplification of MDR-1 mRNA from a preparation of isolated bovine growth plate chondrocytes. Although

TABLE IV. Immunohistochemical Data: Chondrosarcomas*

Patient	C219	C494
Chondrosarcomas Grade I		
22a	2+	1+
22b	1+	2+
23a	1+	1+
23b	1+	1+
24	1+	2+
25	1+	
26a	1+	2+
26b	2+	2+
26c	1+	
26d	1+	
27	1+	1+
Chondrosarcomas Grade II		
28a	3+	4+
28b	1+	3+
29	4+	3+
30a	2+	3+
30b	2+	3+
31	1+	
32	1+	
33	1+	3+
34	3+	
35	1+	2+
Chondrosarcomas Grade III		
36	3+	4+
37	4+	
38a	2+	2+
38b	4+	4+
39a	1+	2+
39b	4+	4+

*Each patient has been given a separate number. If more than one specimen was studied for a given patient (i.e., primary lesion, and then recurrence or metastasis), the consecutive specimens were labeled, a, b, c, etc. C219 and C494 immunoreactivities were evaluated without prior knowledge of the histologic diagnosis. The lesions were graded semiquantitatively on a scale of 0–4+ as described in Table I.

the function of P-glycoprotein in normal cartilage is not evident from the present study, localization to the hypertrophic region of the growth plate, where marked changes occur in the extracellular environment, suggests an excretory or protective function in normal chondrocytes. The expression of P-glycoprotein in cartilage tumors is consistent with the high level of expression typically observed in other tumors derived from tissue normally expressing this protein [43,44].

Although the functional importance of P-glycoprotein as a drug resistance mechanism was first described in hematopoietic and epithelial malignancies [16–20], its role in chemotherapeutic resistance in primary osseous and soft tissue sarcomas has been increasingly appreciated [21–27]. Wunder et al. [25] reported varying levels of MDR-1 mRNA in 18 osteosarcomas analyzed by Northern blot and found a trend toward worse outcome in patients with tumors exhibiting high levels of MDR-1 expression [25]. Rosier et al. [26] found that P-glycoprotein immunoreactivity in osteosarcoma inversely correlated with histologic response to chemotherapy and appeared to correlate with metastasis. Bramlett and Lennington [27] reported P-glycoprotein expression in 14 of 21 osteosarcomas and 8 of these 14 cases showed staining only in foci demonstrating chondroblastic differentiation.

Initiation of P-glycoprotein expression following chemotherapy has been described in osteosarcomas following chemotherapy [26]. Several mechanisms are likely in the regulation of P-glycoprotein, although the development of drug resistance most likely occurs through the selection of clones of P-glycoprotein positive cells in the presence of cytotoxic agents [45–47]. Other studies have demonstrated induction of P-glycoprotein by chemotherapeutic drugs that act as substrates for this protein transporter [47]. Although the two metastatic tumors in this study that had prior exposure to chemotherapeutic agents also had increased expression of P-glycoprotein, the data are not sufficient to make conclusions regarding an association between chemotherapy and P-glycoprotein expression in cartilage tumors.

The highest levels of P-glycoprotein expression in cartilage tumors occurred in the more malignant tumors, consistent with the pattern of expression in other high grade sarcomas [21,25,26]. In contrast, carcinomas typically have higher levels of P-glycoprotein expression in more differentiated tumors. Similar to the pattern observed in other tumors, the cartilage tumors demonstrated a mixed pattern of P-glycoprotein expression, with intermixed clusters of positive and negative cells [48]. P-glycoprotein has been shown to be regulated by several proto-oncogene transcription factors, including c-fos and c-jun [49–51]. Since c-fos is expressed at high levels in cartilage [52] and overexpression has been implicated in the pathogenesis of chondrosarcomas [53], transcriptional up-regulation of MDR-1 by this factor may be one explanation for the high level of P-glycoprotein found in chondrosarcomas.

P-glycoprotein expression in chondrosarcoma was confirmed through utilization of three separate antibodies to P-glycoprotein, C219, C494, and JSB-1. C219 detects a carboxy terminal intracellular epitope common to both MDR-1 and MDR-2, whereas C494 and JSB1 recognize separate intracellular epitopes of the MDR-1 gene product [29–32]. Although these antibodies have been demonstrated to have some cross-reactivity to other molecules, including C219 with muscle myosin and C494 with pyruvate carboxylase [41,54], the similarity of staining with the three antibodies substantiates the expression of P-glycoprotein in cartilage tumors. Although immunodetection is more commonly performed on frozen tissue sections, similar patterns of staining were observed in experiments comparing frozen and paraffin-embedded tissues, validating the use of archival tissue specimens. The immunohistochemical findings were fur-

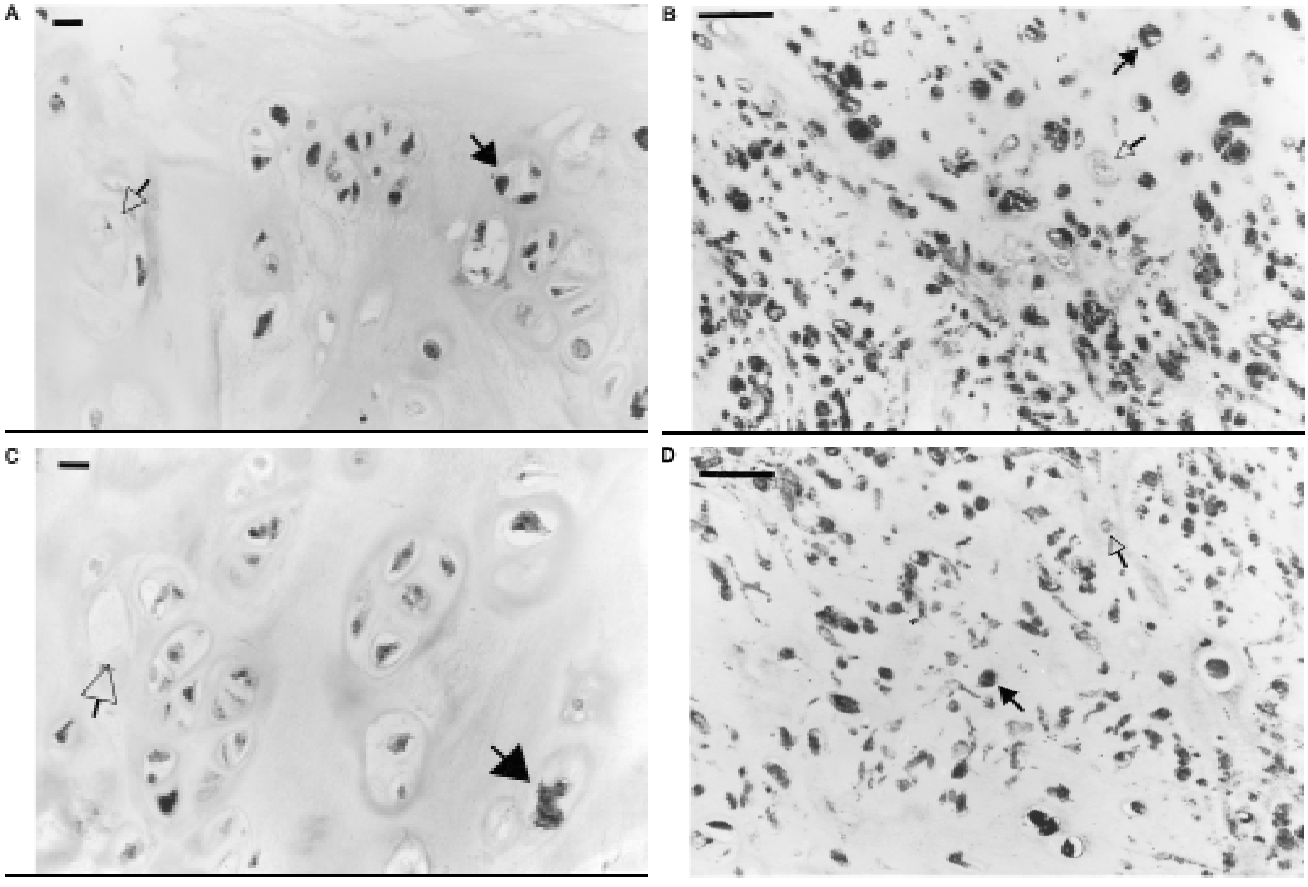

Fig. 4. Immunostaining of a low grade chondrosarcoma and high grade chondrosarcoma for P-glycoprotein. Sections of a low grade and high grade chondrosarcoma were immunostained as described in Materials and Methods using the C219 antibody, (low grade, **A,** original magnification = 400×, bar = 10 μm, and high grade, **B,** original magnification = 200×, bar = 50 μm); the C494 antibody (low grade, **C,** original magnification = 400×, bar = 10 μm and high grade, **D,** original magnification = 200×, bar = 50 μm). P-glycoprotein negative cells are indicated by closed arrows and P-glycoprotein positive cells by solid arrows. Note the similar pattern of staining with the two different antibodies.

Table V. Correlation of Immunoreactivity of C219 and C494 With In Situ Hybridization for MDR-1

		Immunohistochemistry		In situ hybridization		
Patient #	Diagnosis[a]	C219	C494	poly-dT	MDR	sense MDR
23	CS Grade I	1+	1+	4+	1+	—
24	CS Grade I	1+	2+	4+	3+	—
26	CS Grade I	2+	2+	3+	1+	—
27	CS Grade I	1+	1+	3+	1+	—
28	CS Grade II	3+	4+	4+	2+	—
30	CS Grade II	2+	3+	4+	2+	—
33	CS Grade II	1+	3+	4+	1+	—
38	CS Grade III	2+	2+ (primary)	4+	3+	—
39	CS Grade III	4+	4+ (metastasis)	4+	3+	—
13	EC	1+	1+	4+	3+	—

*Each patient has been given a separate number (see Tables II and III). C219 and C494 immunoreactivity and in situ hybridization were evaluated without prior knowledge of the histologic diagnosis. The lesions were graded semiquantitatively on a scale of 0–4+ as described in Table I.

[a]CS = chondrosarcoma; EC = enchondroma.

ther confirmed by in situ hybridization studies. Although two isoforms of MDR have been identified in humans (MDR-1 and MDR-2), drug resistance has been associated only with the expression of the MDR-1 gene [55].

Because of the prognostic importance of P-glycoprotein expression in many tumors, strategies to inhibit expression or function of this protein are currently under investigation. The calcium channel blocker vera-

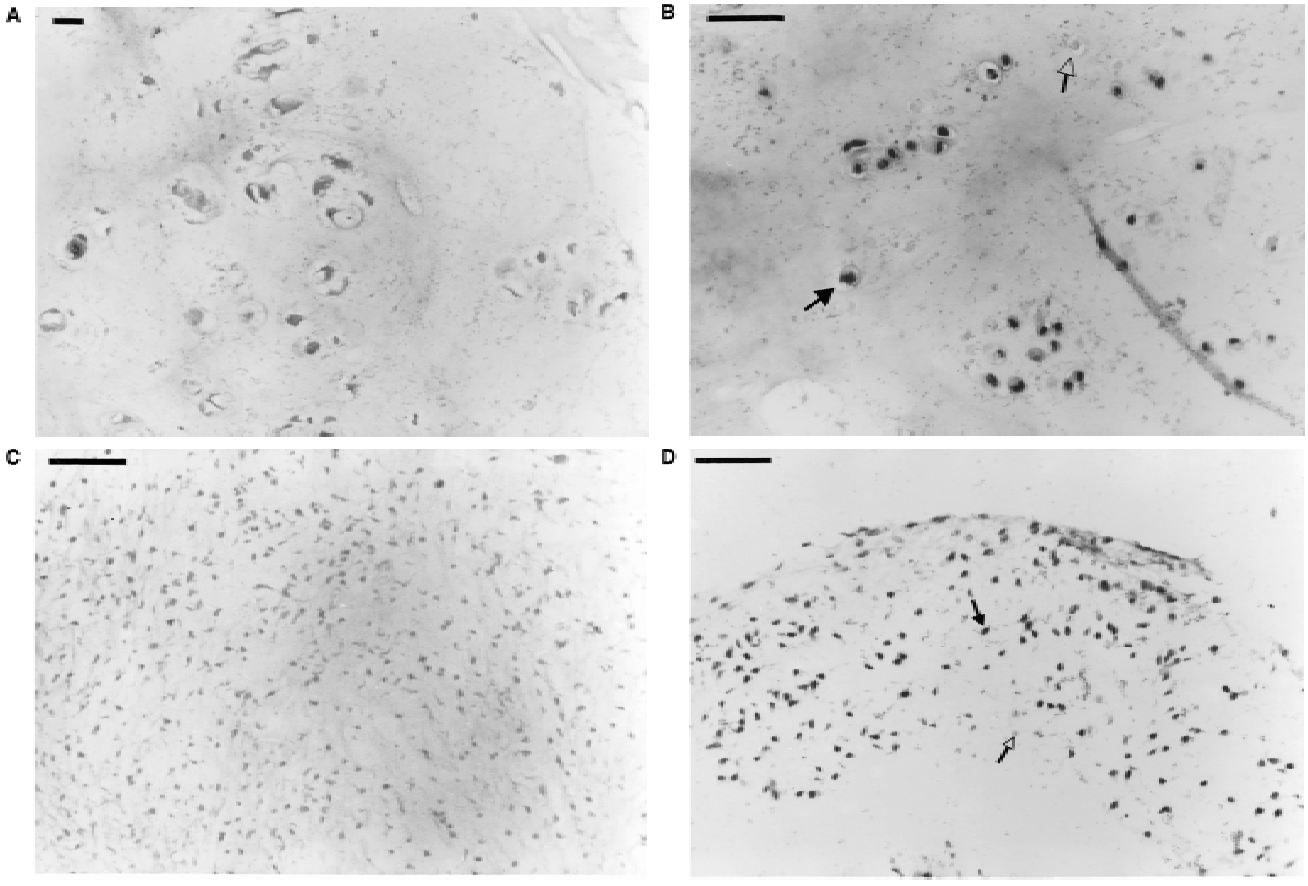

Fig. 5. In situ hybridization of a well-differentiated (grade I) and an intermediate (grade II) chondrosarcoma for MDR-1. In situ hybridization was performed on chondrosarcoma sections as described in Materials and Methods using a digoxigenin-labeled, 30-base probe complementary to MDR-1. Low background staining is demonstrated for both the low grade (**A,** original magnification = 400×, bar = 10 μm) and intermediate grade (**C,** original magnification = 200×, bar = 50 μm) tumors hybridized to a nonsense probe without known homology. In contrast, reactivity was observed in both tumors with more intense staining observed in the intermediate grade tumor (**D,** original magnification = 200×, bar = 50 μm) compared with the low grade chondrosarcoma (**B,** original magnification = 200×, bar = 50 μm). MDR-1 negative cells are indicated by closed arrows and MDR-1 positive cells by solid arrows.

pamil reverses P-glycoprotein-mediated drug resistance, but only at toxic concentrations [56]. Similarly, cyclosporine A is a substrate for P-glycoprotein and its binding to this molecule inhibits the extracellular transport of chemotherapeutic agents and reverses drug resistance [57]. Although clinical trials are currently underway, the future development of less toxic analogs will expand this approach [58].

The present findings suggest that the chemotherapeutic resistance of chondrosarcomas may be mediated through P-glycoprotein expression and provide a potential strategy for the systemic treatment of high grade and disseminated tumors. Through the use of P-glycoprotein inhibitors as adjuncts to conventional chemotherapeutic agents, chondrosarcomas may be rendered chemosensitive, and perhaps this rare tumor should be considered for multicenter clinical trials of P-glycoprotein inhibitors. Whereas P-glycoprotein inhibitors have limited clinical usefulness at the present time, their efficacy should improve as the mechanisms of induction, regulation, and structure-function relationships of the P-glycoprotein molecule are elucidated.

CONCLUSIONS

Immunohistochemical and *in situ* hybridization techniques demonstrated MDR-1 expression in each of 50 benign and malignant cartilage tumors studied, with increased expression in high grade malignancies. The findings may explain the well-recognized lack of responsiveness of chondrosarcomas to chemotherapy.

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